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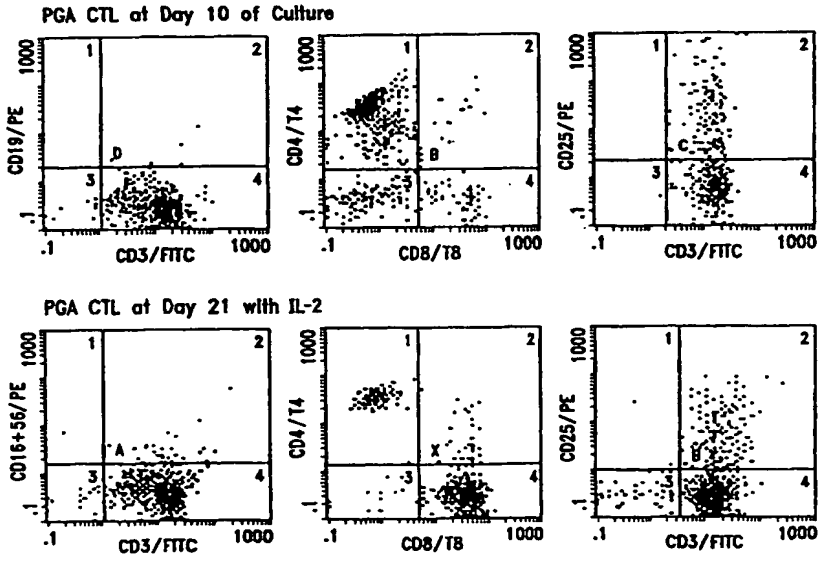

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(57) Abstract

The disclosed invention encompasses methods for obtaining compositions comprising cell populations enriched for cancer–specific, cytotoxic T–lymphocytes. The methods comprise the steps of obtaining cancer cells from a patient, obtaining leukocytes from the patient, and co–culturing the cancer cells with the leukocytes to allow outgrowth of a cancer–specific cytotoxic T–lymphocyte (CSCTL) population. In certain embodiments, the method includes the additional step of inducing an anti–cancer immune response in the patient. Substantially pure populations of CSCTLs are provided that are cytotoxic toward autologous cancer cells, and particularly suitable for treating glioblastomas and other solid tumors. This invention further provides methods for treating cancer in a patient using cellular compositions of the invention, and use of cellular compositions for preparing medicaments for cancer treatment.

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A CELL STRAIN WITH ACTIVATED ANTI-CANCER CYTOTOXIC ACTIVITY

RELATED APPLICATIONS

This application claims the priority benefit of U.S. Provisional Patent Application No. 60/044,601, filed April 23, 1997. The aforementioned priority application is hereby incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to the field of cancer treatment. More specifically, it provides expanded populations of T-lymphocytes that are cytotoxic for autologous tumor cells.

BACKGROUND OF THE INVENTION

In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of cancer treatment, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Failure occurs either because the initial tumor is unresponsive, or because of recurrence due to regrowth at the original site and/or metastases. Even in cancers such as breast cancer where the mortality rate has decreased, successful intervention relies on early detection of the cancerous cells. The etiology, diagnosis and ablation of cancer remain a central focus for medical research and development.

Neoplasia resulting in benign tumors can usually be completely cured by removing the mass surgically. If a tumor becomes malignant, as manifested for example by invasion of surrounding tissue or by the presence of distant metastases, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much more difficult to eradicate all malignant cells.

The three major cancers, in terms of morbidity and mortality, are colon, breast and lung. New surgical procedures offer an increased survival rate for colon cancer. Improved screening methods increase the detection of breast cancer, allowing earlier, less aggressive therapy. Lung cancer remains largely refractory to treatment.

Excluding basal cell carcinoma, there are over one million new cases of cancer per year in the United States alone, and cancer accounts for over one half million deaths per year in this country. In the world as a whole, the most common cancers are those of the liver, lung, stomach, colon/rectum, breast, and uterine cervix, and the total number of new cases per year is over 6 million. Overall, about half the number of people who develop cancer die of it.

Melanoma is one of the human diseases for which there is an acute need of new therapeutic modalities. It is a particularly aggressive form of skin cancer, and occurs in increased frequency in individuals with regular unguarded sun exposure. In the early disease phases, melanoma is characterized by proliferation at the dermal-epidermal junction, which soon invades adjacent tissue and metastasizes widely. Once it has metastasized, it is often impossible to extirpate and is consequently fatal. Worldwide, 70,000 patients are diagnosed with melanoma and it is responsible for 25,000 reported deaths each year. The American Cancer Society projects that by the year 2000, 1 out of every 75 Americans will be diagnosed with melanoma.

Neuroblastoma is a highly malignant tumor occurring during infancy and early childhood. Except for Wilm's tumor, it is the most common retroperitoneal tumor in children. This tumor metastasizes early, with widespread involvement of lymph nodes, liver, bone, lung, and bone marrow. While the primary tumor is resolvable by resection, the recurrence rate is high.

Each year, approximately 15,000 cases of high grade astrocytomas are diagnosed in the United States. These numbers are growing in both pediatric and adult populations. Standard treatments for such tumors include cytoreductive surgery followed by radiation therapy or chemotherapy. There is no cure, and virtually all patients ultimately succumb to recurrent or progressive disease. The overall survival for grade IV astrocytomas (glioblastoma multiforme) is poor, with ~50% of patients dying in the first year after diagnosis. Since these tumors are aggressive and highly resistant to standard treatments, new therapies are needed.

Small cell lung cancer is the most malignant and fastest growing form of lung cancer and accounts for 20-25% of new cases of lung cancer. 60,000 cases will be diagnosed in the U.S. in 1996. The primary tumor is generally responsive to chemotherapy, but is usually quickly followed by wide-spread metastasis. The median survival time at diagnosis is approximately 1 year, with a 5 year survival rate of less than 5%.

Breast cancer is one of the most common cancers and is the third leading cause of death from cancers in the United States with an annual incidence of about 182,000 new cases and nearly 50,000 deaths. In the industrial nations, approximately one in eight women can expect to develop breast cancer. The mortality rate for breast cancer has remained unchanged since 1930. It has increased an average of 0.2% per year, but decreased in women under 65 years of age by an average of 0.3% per year. Preliminary data suggest that breast cancer mortality is beginning to decrease, probably as a result of increased diagnoses of localized cancer and carcinoma *in situ*. See e.g., Marchant (1994) Contemporary Management of Breast Disease II: Breast Cancer, in: *Obstetrics and Gynecology Clinics of North America* 21:555–560; and Colditz (1993) *Cancer Suppl.* 71:1480–1489.

Non-Hodgkin's B cell lymphomas are cancers of the immune system that are expected to afflict approximately 225,000 patients in the United States in 1996. These cancers are diverse with respect to prognosis and treatment, and are generally classified into one of three grades. The median survival of the lowest grade is 6.6 years and the higher grade cancers have much lower life

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expectancy. Virtually all non-Hodgkin's B cell lymphomas are incurable. New diagnoses of non-Hodgkins lymphomas have increased approximately 7% annually over the past decade, with 52,700 new diagnoses estimated for 1996. The increase is due in part to the increasing prevalence of lymphomas in the AIDS patient population.

In spite of the difficulties, effective cures using anticancer drugs (alone or in combination with other treatments) have been devised for some formerly highly lethal cancers. Most notable among these are Hodgkin's lymphoma, testicular cancer, choriocarcinoma, and some leukemias and other cancers of childhood. For several of the more common cancers, early diagnosis, appropriate surgery or local radiotherapy enables a large proportion of patients to recover.

Current methods of cancer treatment are relatively non-selective. Surgery removes the diseased tissue, radiotherapy shrinks solid tumors and chemotherapy kills rapidly dividing cells. Chemotherapy, in particular, results in numerous side effects, in some cases so severe to preclude the use of potentially effective drugs. Moreover, cancers often develop resistance to chemotherapeutic drugs.

Numerous efforts are being made to enhance the specificity of cancer therapy. For review, see Kohn and Liotta (1995) *Cancer Res.* 55:1856-1862. In particular, identification of cell surface proteins expressed exclusively or preferentially on certain tumors allows the formulation of more selective treatment strategies. Antibodies directed to these antigens have been used in immunotherapy of several types of cancer.

Numerous phase I clinical trials have been performed to assess the feasibility and toxicities of a variety of immune-enhancing therapies, including intravenous administration of recombinant cytokines such as IL-2, interferon and TNF-α, nonspecific immune enhancing agents, and various monoclonal antibodies. While none of these trials was associated with serious clinical complications, only occasional and isolated reports of complete responses were noted. Long term follow-up studies failed to demonstrate durable long term responses. Few, if any, studies have shown the induction of tumor-specific immunity in patients receiving such treatments.

Adoptive immunotherapy, the infusion of immunologically active cells into a tumor-bearing host in order to effect tumor regression, has been an attractive approach to the therapy of cancer for several decades. Two general approaches have been pursued. In the first, donor cells are collected that are either naturally reactive against the host's tumor, based on differences in the expression of histocompatibility antigens, or made to be reactive using a variety of "immunizing" techniques. Cells from this donor are then transfused to a tumor-bearing host. In the second general approach, cells from a tumor-bearing host are collected, activated *ex vivo* against the tumor and then reinfused into the host. Triozzi (1993) *Stem Cells* 11:204-211; and Sussman et al. (1994) *Annals Surg. Oncol.* 1:296.

The rationale for cellular immunotherapy of cancer derives from animal models indicating that cellular and not humoral immune responses were responsible for tumor regression. There are a number of potential advantages to using activated cells for cancer treatment in humans. Cancer

patients are often immunosuppressed either because of the underlying malignancy or as a result of therapy. Reinfusion of immune effector cells provides a means to boost a suppressed cellular response. Cells can also be relatively specific effectors. Immunotherapy with, for example, cytokines, sets off a cascade of both beneficial and deleterious, specific and non-specific, antitumor responses. Because of the presence of recognition structures and a highly regulated release of effector molecules, the infusion of cells can effect a more specific antitumor response. Viable immune cells are also a potentially self-renewing source of anti-tumor activity *in vivo*. In addition, immunotherapy offers the potential of manipulating, *ex vivo*, factors that may be suppressive *in vivo*.

Various effector cells have been analyzed for their potential to serve specific anti-tumor roles. Among them are natural killer (NK) cells, which appear to be the source of lymphokine-activated killer (LAK) activity; peripheral blood leukocytes (PBL); and tumor-infiltrating lymphocytes (TIL), which are predominantly of the $CD8^+$ phenotype. TIL are generated by the culture of lymphoid cells dissociated from solid tumors in high concentrations of IL-2. It was found that immunotherapy with TIL significantly prolonged survival in mice with advanced pulmonary metastases that were refractory to LAK cells. Rosenberg et al. (1986) *Science* 233:1318. In contrast to LAK cells, TIL have been shown to have relatively more specific immunoreactivity against the tumor of origin in *in vitro* cytotoxicity assays; this tumor specificity was associated with a 50- to 100-fold increased efficacy in the reduction of lung metastases when compared with LAK cells. Spiess et al. (1987) *J. National Cancer Inst.* 79:1067.

Most of the early studies were performed in animal models. Typically, tumor-draining lymph node (TDLN) cells, TIL, lymph node (LN) cells or splenocytes have been collected, stimulated and expanded *in vitro*, then re-introduced into the tumor-bearing animal. A common *in vitro* manipulation of LN cells, TDLN cells or splenocytes has been the sequential treatment of these cells first with anti-CD3 monoclonal antibody, then with IL-2 to effect stimulation and expansion of cytotoxic T cells. Matsumura et al. (1993) *Cancer Res.* 53:4315. Crossland et al. (1991) *J. Immunol.* 146:4414; Loeffler et al. (1991) *Cancer Res.* 51:2127. Sussman et al. (1995) *J. Immunother. Emphasis Tumor Immunology* 18:35; and Wahl et al. (1994) *J. Immunother. Emphasis Tumor Immunology* 15:242. In attempts to enhance the effects of anti-CD3 and IL-2, elements have been added to this *in vitro* treatment scheme. For example, LPS-activated B cells were used to provide costimulatory signals in the *in vitro* induction with anti-CD3 and IL-2 of antitumor effector cells from populations of LN or TDLN cells. Okamato et al. (1995) *Cancer Immunol. Immunother.* 40:173. IL-1 added during the anti-CD-3 step, but not during the IL-2 step, led to the generation of cells that were significantly more therapeutically efficacious than cells generated in the absence of IL-1. Hammel et al. (1994) *J. Immunother. Emphasis Tumor Immunol.* 16:1.

It has been reported that direct implantation of allogeneic cytotoxic T-lymphocytes (CTL) simultaneously with a brain cancer cell line in Fischer rats resulted in a significant survival

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advantage, compared with injection of the cancer cells alone, or in combination with over other populations of lymphocytes — including syngeneic CTL, lymphokine-activated killer cells, adherent-LAK cells or IL-2 alone. Kruse et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:9577.

Another approach has been to activate lymphocytes from a tumor-bearing animal with irradiated autologous tumor cells, and then expand the population by restimulation with tumor cells in the presence of concanavalin A-stimulated rat spleen culture supernatant and splenic antigen-presenting cells. Kim et al. (1995) *J. Biochem. Mol. Biol.* 28:556. Bacterial superantigens have been used as stimulatory agents to activate T cells with particular T cell receptor β chain variable regions. Stimulation with various staphylococcal enterotoxins, followed by culture in IL-2 resulted in enhanced tumor rejection by the resulting T cell population. Shu et al. (1994) *J. Immunol.* 152:1277; and Inoue et al. (1996) *Biotherapy* 10:259. In one case, activated cells were shown to contain both CD4+ and CD8+ cells, and, after intravenous administration of the activated cells, 3-day established brain tumors were eradicated. The therapeutic efficacy was greatly enhanced if tumor-bearing mice were also treated with whole-body sublethal irradiation prior to T cell infusion. Inoue et al. (1996) *Biotherapy* 10:259.

Treatment of TDLN with the protein kinase C activator bryostatin 1 and the calcium ionophore ionomycin represents a non-specific method of activation that has been attempted. TDLN were initially cultured with autologous tumor cells in the presence of IL-2, then treated with bryostatin and ionomycin. This regimen resulted in marked *in vitro* proliferation and 15-fold expansion of cell numbers over a 2-week period, while retaining specific cytotoxicity toward autologous tumor cells. Tuttle et al. (1992) *J. Immunother.* 12:75.

Attempts have been made to improve the anti-cancer activity human lymphocytes by in vitro manipulation, including cytokine stimulation, gene therapy, co-cultures of lymphocytes and tumor cells, and antibody stimulation. Cytokine stimulation has been used either alone or in combination with other treatments, such as anti-CD3 antibodies attached to a solid support. For example, recombinant IL-2 (rIL-2) has been used to expand *in vitro* the T cell population, followed by reinfusion of the activated lymphocyte population, usually along with rIL-2. Immobilized anti-CD3 antibodies have been used to activate intracellular signaling pathways that result in synthesis of effector molecules. Sekine et al. (1993) *Biomed. Pharmacother.* 47:73-78; U.S. Patent 4,690,915; Ikarashi et al. (1992) *Japan. J. Cancer Res.* 83:1359-1365; and Goedegebuure et al. (1995) *J. Clin. Oncol.* 13:1939-1949.

Gene therapy, in which effector cells are transduced with genes encoding cytokines to enhance cytotoxic potential has also been attempted. Another gene therapy approach has been to transduce T cells with genes encoding chimeric antibody/T-cell receptors. For example, the T-cell receptor portion of the chimeric molecule activates the T-cell, leading to cytokine release and specific lysis of the target, while the antibody portion consists of a combining site with specificity for a tumor antigen and serves to bring the activated T-cell and its target into the proximity of one another. Hwu et al. (1995) *Cancer Res.* 55:3369; Hwu and Rosenberg (1994) *Cancer Detect.*

Prev. 18:43-50; Abe et al. (1996) *Biochem. Biophys. Res. Comm.* 218:164-170; and Hanaia et al. (1995) *Am. J. Med.* 99:537-552.

Co-culturing of TIL with autologous tumor cells is generally accompanied by cytokine stimulation. Peyret et al. (1991) *Chirurgie* 117:700-709. The TIL are grown from a tumor biopsy suspension using an interleukin-2 (IL-2) medium over a 30-45 day duration (US Patent No. 5,126,132). Suitable numbers were found to be larger than 10^{11}, generated by large scale expansion system of culturing and harvesting. Cell surface phenotypes are found to be mixes of $CD3^+/CD4^+$ or $CD3^+/CD8^+$ lymphocytes. Only melanoma derived TIL were found to be consistently cytolytic, especially those not derived from tumor involved lymph node biopsies. Yannelli et al. (1996) *Int. J. Cancer* 65:413.

TIL grown from colon carcinoma specimens in IL-2 contained mixed populations of $CD4^+$ and $CD8^+$ cells. Fresh cryo-preserved colon tumors were not lysed by autologous TIL, and poorly lysed by lymphokine-activated killer (LAK) cells. However, four of ten TIL secreted cytokines specific to HLA-matched allogeneic carcinomas only, not melanomas or HLA-unmatched colon carcinomas. This provides evidence for specific immune recognition of a potentially conserved or shared tumor antigen by human colon carcinomas. Hom et al. (1993) *Cancer Immunol. Immunother* 36:1.

A randomized study of IL-2 with and without LAK cells involving patients of melanoma and renal cell cancer suggested a trend toward increased survival in melanoma patients with both IL-2 and LAK cells, but no trends in patients of renal cell cancer. Rosenberg et al. (1993) *J. Natl. Cancer Inst.* 85:622. Further studies of T cells cultured in IL-2 have yielded cells functionally similar to LAK cells from peripheral blood mononuclear cells. Surface marker examination for $CD4^+$ determination of the two cells show differing activation marker expression. This suggests the TIL from the renal cell carcinomas are activated. However, CD25 expression was not different, suggesting TIL have a block in proliferative capacity. General failure of these TIL to be tumor-specific may be due to failure to grow in IL-2. Alexander et al. (1995) *J. Immunother. Emphasis Tumor Immunol.* 17:39.

TIL derived from a few breast cancer patients were cultured, of which the $CD4^+$ TIL specifically released cytokines when stimulated by autologous tumor and not by a large panel of other stimulators. The findings demonstrated that tumor-associated antigens can be detected in some breast cancer patients. Dadmarz et al. *Cancer Immunol. Immunother* 40:1 (1995).

After more successful trials with melanoma than other cancers, TIL causing tumor regression were established and melanoma antigens were identified. Since melanomas from different patients have been shown to express shared tumor antigens, melanoma-specific cytotoxic T-lymphocytes (CTL) cultures derived from TIL were tested on histologies including retinoblastoma, neuroblastoma, neuroepithelioma, astrocytoma, neuroglioma and Ewing's sarcoma. The analysis demonstrated cross-reactivity between melanomas and Ewing's sarcomas. This has implications for CTL-defined tumor antigen development and raises the possibility of

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specific antitumor therapies to be reactive against more than one kind of cancer. Shamamian et al. (1994) *Cancer Immunol. Immunother* 39:73. Of the TIL derived from melanoma patients, mostly the $CD8^+$ cells have been found to be cytolytic, although some $CD4^+$ cells have also shown some cytolytic activity. Markus et al. (1995) *J. Interferon Cytokine Res.* 15:739.

There are gene therapy studies in related areas involving genetic modification. These include modified tumors, TIL modified by chimeric receptor genes and in-depth research into the identification, cloning and TIL recognition of gp100, a melanocyte lineage-specific antigen associated with metastatic melanoma tumor regression. Salgaller et al. (1995) *Cancer Res.* 55:4972.

Erythropoietin (EPO) may be used as a relatively non-toxic substitute for IL-2, which is coadministered with transduced TIL. Data suggest that EPO binding causes the activation of the IL-2 signal pathway. Minamoto et al. (1995) *Blood* 86:2281. Anti-idiotypic antibodies, wherein the anti-idiotype is generated against an antibody specific for a tumor antigen, has been used to stimulate lymphocytes *in vitro*. U.S. Patent 4,918,164. Bi-specific antibodies are created in which one antibody combining site recognizes and binds to a T-cell surface antigen such as CD3 and the second combining site serves to juxtapose the T-cell with a tumor cell by virtue of its specificity for a tumor surface antigen. Bolhuis et al. (1991) *Cancer Immunol. Immunother.* 34:1-8.

Although a degree of success has been claimed using some of these methods of *ex vivo* activation, several problems still exist. For instance, the specificity of the activated lymphocytes is heterogeneous, such that only a proportion of the activated cell population to be re-introduced into the patient is specific for the tumor; the percentage of the *ex vivo*-activated cells that traffic to the site of the tumor is low (Abe et al. (1996) *Biochem. Biophys. Res. Comm.* 218:164-170); use of high levels of rIL-2 can be toxic, and furthermore tends to select for a population of IL-2-dependent cells; and, most significantly, a low frequency of objective response has generally been observed. For example, in a study of sixteen patients with metastatic melanoma or renal cell carcinoma, after treatment with *in vitro*-IL-2-activated TIL, only 19% showed a complete response, 56% showed no response and 25% had progressive disease. Goedegebuure et al. (1995) *J. Clin. Oncol.* 13:1939. Similarly, in a study of 24 patients with non-small-cell lung cancer treated with TIL that had been activated *in vitro* with rIL-2, the median survival was 14 months, with a two-year survival rate of 40%. Ratto et al. (1995) *J. Thorac. Cardiovasc. Surg.* 109:1212. In a clinical trial of adoptive immunotherapy, carried out with PBL cocultured *in vitro* with autologous tumor cells and IL-2, and reinfused into patients with advanced melanoma, no major therapeutic response was observed. Sporn et al. (1993) *Cancer Immunol. Immunother.* 37:175.

Treatment using currently available cell compositions has failed to impact many other types of cancer, including breast cancer, colon cancer and brain tumors. In addition, the collection of material for preparation of TIL is difficult and cumbersome, and not amenable to a number of the important cancer types.

It would clearly be an advantage if a rapidly effective cell population could be produced from a more convenient source — such as the peripheral blood of the cancer patients. A few preliminary studies have been done with this in mind, with a view towards the treatment of melanoma.

Stevens et al. reported generation of tumor-specific CTLs from melanoma patients using peripheral blood stimulated with tumor cell lines. Mixed lymphocyte tumor cell cultures (MLTCs) were established using patient peripheral blood leukocytes (PBL) and HLA-matched tumor cell lines, and exogenous IL-2 and IL-4. CTL lines were obtained from a proportion of patient samples, and showed specificity for histocompatibility antigens and defined melanoma antigens. Use of these cells in treatment was not reported in the article. Stevens et al. (1995) *J. Immunol.* 154:762.

Crowley et al. reported generation of human autologous melanoma-specific cytotoxic T cells, again by culturing PBL with HLA class I matched allogeneic melanoma cell lines. The CTL were predominantly CD8+, and proliferated in response to both autologous tumor and class I matched melanomas, but not with class I matched non-melanoma lines. Use of these cells in treatment was not reported in the article. Crowley et al. (1990) *Cancer Res.* 50:492.

Celis et al. reported induction of anti-tumor CTLs in normal humans using primary cultures and synthetic peptide epitopes. Peripheral blood mononuclear cells (PBMC) were prepared using blood from normal volunteers. Synthetic nonapeptides from the melanoma antigen MAGE-2 and MAGE-3 were presented via irradiated lymphoblastoid antigen presenting cells to CD4+ cell depleted responder cells. The cells were then cultured in the presence of IL-2 for 19 days, and tested for CTL activity. Specific cytotoxicity was observed and determined to be MHC class I restricted. Use of these cells in treatment was not reported in the article. Celis et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:2105.

Sporn et al. reported adoptive immunotherapy using PBL cocultured in vitro with autologous tumor cells and IL-2. PBL from each of 14 patients were cocultured with autologous irradiated melanoma cells for 7 days, and then expanded in IL-2 containing medium for up to 28 days. The lymphocytes were then returned to the patient along with intravenous IL-2 and cyclophosphamide. *No* major therapeutic response was observed in the study, except for a degree of toxicity attributed to the IL-2. Sporn et al. (1993) *Cancer Immunol.. Immunother.* 37:175.

None of these studies indicated an ability of the cells to home to the area of the tumor, which is presumably required for them to have any beneficial effect. Therapy of brain cancers is even more uncertain, since they tend to be more sequestered, and have less well defined tumor antigens.

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DISCLOSURE OF THE INVENTION

The present invention encompasses methods of obtaining compositions comprising cell populations enriched for cancer-specific cytotoxic T-lymphocytes. The methods comprise obtaining leukocytes from a cancer patient who has an immune response to the patient's own tumor cells, obtaining cancer cells from the patient before or after obtaining the leukocytes, co-culturing the leukocytes and cancer cells for a duration and under conditions sufficient to allow outgrowth of a cancer-specific cytotoxic T-lymphocyte (CSCTL) population. The CSCTLs can be maintained and passaged in culture on an ongoing basis and retain tumor specificity for the patient's tumor. The invention further encompasses methods of obtaining CSCTLs from a patient in whom an immune response to the patient's tumor cells has been induced, for example, by vaccination or cytoimplantation. In a preferred embodiment, the inducing of the anti-cancer response in the patient comprises the step of implanting lymphocytes into a tumor bed of the patient, either directly into the tumor or into the space formed by surgical resection of the tumor, wherein the lymphocytes are obtained from a different donor and have been alloactivated against leukocytes of the patient.

In particular, the methods encompassed by the present invention are suitable for treating glioblastomas or other solid tumors. The tumor cells with which the patient's leukocytes are co-cultured can be transformed with expression vectors that allow the synthesis of therapeutic proteins. The methods are useful for generating CSCTLs with specific cytotoxic activity against autologous cancer cells. Accordingly, the present invention also encompasses CSCTLs obtained by any of the methods of the present invention.

The invention further encompasses substantially pure populations of CSCTLs which are cytotoxic toward autologous tumor cells.

The invention encompasses methods of treating glioblastomas or other cancers in patients, comprising administering a therapeutically effective amount of the CSCTLs. Preferably, the cells are administered *in situ* to the tumor. Alternatively, the cells are administered systemically. The increase in immunologic reaction can inhibit tumor growth, decrease size and/or eradicate established tumors or developing metastases, as well as inhibit recurrence at the site of surgically debulked tumors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph pertaining to reculturing of CTL developed from leukocytes of a cancer patient that are specific for autologous brain cancer cells. Results show the dependence of T cell growth on the presence of both IL-2 and tumor antigens against which the cells are previously cultured. Peripheral blood leukocytes were cultured in growth medium 1) without IL-2 (diamonds; broken lines); 2) with 100 U/ml IL-2 (squares; broken lines); 3) in the presence of autologous tumor

(JTBT) (triangle; broken lines); or 4) in the presence of autologous tumor plus 100 U/ml IL-2 (diamonds; solid lines).

Figure 2 is a graph pertaining to a test culture for CTL developed from leukocytes of a cancer patient that are specific for two unrelated (third party) brain cancer cells: U373 (Upper panel), or ACBT (Lower panel). Results show the dependence of T cell growth on the presence of both IL-2 and tumor antigens against which the cells are previously cultured.

Figure 3 is a graph pertaining to a preparative 30-day culture for human CTL specific for autologous tumor cells. Pulsing with fresh tumor cells (Ag) and 100-200 units IL-2 is shown. Between about 1 and 2 x 10^9 cells were harvested at the end of the 30 day period.

Figure 4 is a chart pertaining to the functional characterization of human anti-tumor CTLs. Preparative cultures of CTLs were obtained using PGA (autologous) stimulators, U373 (third party) stimulators, or ACBT (third party) stimulators. This chart shows the results of a 4 hour ^{51}Cr release assay. Boxed numbers indicate specific killing of the brain cancer cell line against which the respective cell population was raised. Also shown are the ability of anti-class I or anti-class II antibodies to block the cytotoxicity, consistent with MHC restricted lysis.

Figure 5 is a six-panel spot graph pertaining to the phenotypic characterization of human anti-tumor CTLs, as determined by FACS analysis. Preparative culture of CTL against autologous tumor cells were sampled at about 10 days and at about 21 days. The phenotype shifts from predominantly CD4+ cells to predominantly CD8+ cells.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention provides methods for obtaining compositions enriched for cancer-specific cytotoxic lymphocytes (CSCTLs). In certain embodiments of this invention, the cancer is a glioblastoma. The methods include obtaining leukocytes from a cancer patient and co-culturing the leukocytes with tumor cells derived from the patient for a duration and under conditions sufficient to selectively expand a population that is cytotoxic toward autologous tumor cells. These cells are substantially free of other types of cells and are termed cancer-specific cytotoxic T-lymphocytes (CSCTLs). Also provided are cell compositions obtained by the preparation methods and methods of treatment using the compositions.

The term "T-lymphocytes" as used herein denotes lymphocytes that are phenotypically CD3+, typically detected using an anti-CD3 monoclonal antibody in combination with a suitable labeling technique. The T-lymphocytes of this invention are also generally positive for CD4, CD8, or both.

The term "specific cytotoxic activity" as used herein means that the CSCTLs specifically lyse the tumor cells with which they are cultured and not normal, non-cancerous cells from the same patient or other target cell donor. The CSCTLs on occasion may cross-react with tumors

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from other individuals expressing related tumor-associated antigens. Cytotoxicity toward cancer cells can be measured by any means known in the art, *e.g.*, by standard ^{51}Cr-release assay.

The term "cancer cell", used either in the singular or plural form, refers to cells that have undergone a malignant transformation that makes them pathological to the host organism. Primary cancer cells (that is, cells obtained from near the site of malignant transformation) can be readily distinguished from non-cancerous cells by well-established techniques, particularly histological examination. The definition of a cancer cell, as used herein, includes not only a primary cancer cell, but any cell derived from a cancer cell ancestor. This includes metastasized cancer cells, and *in vitro* cultures and cell lines derived from cancer cells. When referring to a type of cancer that normally manifests as a solid tumor, a "clinically detectable" tumor is one that is detectable on the basis of tumor mass; *e.g.*, by such procedures as CAT scan, magnetic resonance imaging (MRI), X-ray, ultrasound or palpation. Biochemical or immunologic findings alone may be insufficient to meet this definition.

The present invention provides methods for obtaining a cell population enriched for T-lymphocytes with specific cytotoxic activity against autologous cancer cells, comprising the steps of: a) obtaining cancer cells from a patient, b) obtaining a leukocyte population comprising T-lymphocytes from the patient at a time when the patient has an anti-cancer immune response, and c) culturing the leukocytes with the cancer cells for a time and under conditions sufficient to allow outgrowth of the T-lymphocytes. Step a) can occur before, during, or after step b).

Tumor cells are typically obtained from a cancer patient by resection, biopsy, or endoscopic sampling; the cells may be used directly, stored frozen, or maintained or expanded in culture. Samples of the both the tumor and the patient's blood or blood fraction should be thoroughly tested to ensure sterility before co-culturing of the cells. Standard sterility tests are known to those of skill in the art and are not described in detail herein. The tumor cells can be cultured *in vitro* to generate a cell line, as exemplified in the examples below. Conditions for reliably establishing short-term cultures and obtaining at least 10^8 cells from a variety of tumor types is described in Dillmar et al. (1993) *J. Immunother.* 14:65-69. Alternatively, tumor cells can be dispersed from, for example, a biopsy sample, by standard mechanical means before use.

Preferably, subjects from which the leukocytes are obtained have an active systemic immune response to the tumor. An immune response to the tumor may be spontaneous, *i.e.*, occurring without being elicited by introduction of exogenous substances that would evoke an immune response toward the cancer cells. Alternatively, a tumor-specific immune response may be induced or boosted by administration of an immunogenic composition in one or more doses.

Various methods of eliciting an active immune response are known in the art or are under current development. For example, the following immunogenic compositions can be administered. 1) Anti-idiotypic antibodies. Nelson et al. (1996) *Blood* 88:580-589; Stevenson et al (1995) *Immunological Reviews* 145:211-218; 2) carcinoembryonic antigen. Conry et al. (1995) *Gene Therapy* 2:59-65; 3) mucin antigens such as MUC-1. Barratt-Boyes (1996) *Cancer Immunology*

and Immunotherapy 43:142-151; 4) MAGE-1 antigen Hoon et al. (1995) *J. Immunology* 154:730-737; 5) MART-1 antigen. Marincola et al (1996) *J. Immunother. Emphasis Tumor Immunol.* 19:266-277; 6) cocktails of allogeneic melanoma cells. Morton et al. (1996) *Cancer Journal for Clinicians* 46:225-244.

Preferred methods for raising or boosting an anti-tumor immunological response is administration of immunogenic compositions of activated cells either directly into the tumor bed, or at a peripheral site.

In certain preferred embodiments, the immune response of the patient is stimulated (either de novo or in order to boost the response) by using a cytoimplant technique. This involves implanting lymphocytes into a tumor bed of the patient, wherein the lymphocytes are obtained from a different donor and have been alloactivated against stimulated leukocytes. A population of cells comprising lymphocytes are obtained from a third party donor with different HLA antigens from the subject to be treated, for example, by leukapheresis or collection of whole blood, followed by separation of leukocytes by a suitable technique such as centrifugation through Histopaque®. Lymphocytes in the donor cell population are then alloactivated by culturing in vitro with stimulator leukocytes, so as to cause stimulation of T helper-inducer cells (predominantly CD4+) against alloantigens of the subject.

There are several options for the source of stimulator leukocytes. In one option, the stimulator leukocytes are histocompatible with the patient, and are preferably lymph node cells or peripheral blood mononuclear cells obtained from the patient to be treated. The cells are typically inactivated by such treatments as irradiation or applying mitomycin C. The donor lymphocytes are then stimulated with patient leukocytes in culture. For a more complete description of cytoimplants, their preparation and use, the reader is directed to PCT patent application WO 96/29394, which is hereby incorporated herein by reference in its entirety. In another option, the stimulator leukocytes are obtained from another unrelated third-party donor, typically enriched for mononuclear cells, and again may be inactivated. The lymphocytes from the first donor are then stimulated with leukocytes of the second donor. In a variation of this option, leukocytes or mononuclear cells from a plurality of three or more donors are cultured together without inactivation, so that multiple-way activation occurs. This is described in US patent application [Serial No. pending, attorney docket 22000-30594.00].

Stimulation of the lymphocytes is conducted by way of an in vitro culture. The cell culturing is conducted under conditions sufficient for alloactivation, which is generally from 1-5 days and typically about 48 to 72 hours. Alloactivation can be confirmed by determining cytokine secretion by standard immunoassay or by cytokine receptor expression. The alloactivated cells are then typically washed free of culture medium, placed in a suitable excipient such as isotonic saline or clotted plasma, and then implanted into the tumor bed. The implant may be placed directly into the tumor by injection, or the tumor may be resected and the implant placed into the resulting cavity. Typical amounts of implanted alloactivated cells are about 0.5×10^9 to 20×10^9,

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more preferably between about 2 x 10^9 and 10 x 10^9, depending on the size of the tumor bed and the number of alloactivated cells available. The immunogenic cell population is then administered into a tumor bed of the patient (that is to say, in the vicinity of the tumor with or without removal of the tumor from the site), the cells having been obtained by collecting lymphocytes from a third party donor and alloactivating them against leukocytes of the patient or against leukocytes of another third party donor. An anti-tumor immune response is typically present within a few weeks of treatment.

As an alternative or in addition to immune stimulation by cytoimplant, immunogenic cell compositions may be given peripherally. Exemplary immunogenic cell compositions are described in PCT/US97/18718 ("Cancer Immunotherapy Using Tumor Cells Combined With Mixed Lymphocytes"); and PCT/US97/13205 ("Cancer Immunotherapy using Autologous Tumor Cells combined with allogeneic cytokine-secreting cells"). For example, an immunogenic cell population can be administered to the patient at a site distant from a tumor bed, the cell population comprising cancer cells from the patient or their progeny, and further comprising lymphocytes from a third party donor alloactivated against leukocytes of the patient or another third party donor.

The expression of an active anti-tumor response in the patient can be verified, if desired, by employing a suitable assay. For example, evidence of an antibody response to the tumor can be obtained testing the patient's serum using an immunoassay using whole tumor cells or tumor cell lysates as a source of antigen, overlaying with the serum and detecting bound tumor-specific antibodies with labeled anti-immunoglobulin. Suitable tumor cells would preferably be those from the patient or a cell line derived from the same tissue. In a variation of this assay the antigen source is a tissue section from, for example, a biopsy sample.

A cellular immune response can be demonstrated, for example, by measuring the T cell proliferative activity in cells (particularly PBL) sampled from the subject. Peripheral blood mononuclear cells (PBMC) are isolated from PBL. Tumor cells, preferably derived from the subject and inactivated, for example, by irradiation or mitomycin treatment, are used as stimulators. A non-specific mitogen such as PHA serves as a positive control; incubation with an unrelated stimulator cell, or non-cancerous cell from the subject serves as a negative control. After incubation of the PBMCs with the stimulators for an appropriate period (typically 5 days), [^{3}H]thymidine incorporation is measured. If desired, determination of which subset of T cells is proliferating can be performed using flow cytometry. T cell cytotoxicity (CTL) may also be measured. In this test, an enriched T cell population from the subject are used as effectors in a standard ^{51}Cr release assay. Tumor cells are radiolabeled as targets with about 200 μCi of Na_2 $^{51}CrO_4$ for 60 minutes at 37° C, followed by washing. T cells and target cells (~1 × 10^4/well) are then combined at various effector-to-target ratios in 96-well, U-bottom plates. The plates are centrifuged at 100 × *g* for 5 minutes to initiate cell contact, and are incubated for 4-16 hours at 37°C with 5% CO_2. Release of ^{51}Cr is determined in the supernatant, and compared with targets incubated in the absence of T cells (negative control) or with 0.1% TRITON™ X-100 (positive

control). See, *e.g.*, Mishell and Shiigi, eds. *Selected Methods in Cellular Immunology* (1980) W.H. Freeman and Co.

To prepare the therapeutic T cell population, both leukocytes and tumor cells are obtained from the patient. Leukocytes are obtained from whole blood, leukapheresis, or other suitable source. Erythrocytes are removed and the preparation is preferably enriched for mononuclear cells, for example, by centrifugation through a cell separation medium, such as Histopaque™ 1.077 (Sigma, St. Louis, MO). Before culturing, typically the cells are analyzed to determine the number of live cells per unit volume. This can be performed, for example, by using a stain that differentiates between living and dead cells (such as propidium iodide) and counting the cells in a Neubauer chamber.

A patient's leukocytes are cultured together with histocompatible cancer cells preferably from the same individual. The culturing is conducted for a time and under conditions sufficient to allow outgrowth of T-lymphocytes having specific cytotoxic activity against autologous cancer cells. Generally, the time and conditions are sufficient to obtain from about 10^8 to about 10^{11}, typically from about 5×10^8 to about 2×10^{10} and preferably at least about 1×10^9 CSCTLs. The amount of time necessary to obtain a desired number of cells may be about 30 days, but can also be a longer or shorter period of time, depending on growth conditions, inherent growth characteristics of the cytotoxic cells, and/or other factors. Appropriate conditions for the growth of the cells is generally in an atmosphere of 5%CO_2/95% air, culture medium which provides essential nutrients for growth, *e.g.*, AIM V medium. Other important parameters include the ratio of leukocytes to tumor cells. Preferably, the PBMC:tumor cell ratio is in the range of from about 10:1 to about 10,000:1, more preferably from about 50:1 to about 1,000:1, even more preferably about 100:1.

"Outgrowth" means that the number of T-lymphocytes with cytotoxic activity against cancer cells with which they are cultured is increased, preferably at least about 100-fold, more preferably at least about 1000-fold, still more preferably at least about 10^4-fold in relation to the number of leukocytes used to initiate the culture.

The methods of the present invention yields an enriched population of T-lymphocytes having specific cytotoxic activity toward cancer cells. A "substantially pure" population of CSCTLs means a population that comprises at least about 80%, preferably at least about 90%, more preferably at least about 95% T cells according to cell-surface markers.

The essential activity of the T cells is against the cancer cells of the patient desired to be treated. The anti-cancer activity is specific, in that it reacts against cancer cells in the patient in preference to most non-cancerous cells in the patient. Thus, the cells comprise an activity that is related to a tumor-associated antigen or epitope – i.e., an immunological determinant that is expressed on the tumor cells. The CSCTLs of the invention differ from pan-reactive anti-cancer cells like LAK cells, in that they react with only some populations of cancer cells they are tested against, including those of the patient. In certain embodiments of the invention, the anti-cancer activity of the CSCTL is directed against a tumor-associated determinant that is shared between

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different but related tumors of the same tissue type. In other embodiments of the invention, the activity is directed against a more individual determinant or determinant mixture, such that the patient's tumor cells are suitable targets, but not any one of a plurality of tumor cells of the same type from other individuals. In some embodiments of the invention, the activity is restricted to presentation of the determinant in the context of autologous histocompatibility Class I or Class II antigens, particularly HLA-A or HLA-B. In other embodiments of the invention, the activity is not restricted and will lyse different tumor cells, providing they express essentially the same cancer-related determinant or determinant mixture. Preferably, at least about 30%, more preferably at least about 60%, still more preferably at least about 80%, and even more preferably at least about 95% of the T cells in the population have the specific anti-tumor cell activity. Preferably, at least 10%, more preferably at least about 20%, and still more preferably at least 30% of the CD8+ T cells in the population have anti-cancer cytolytic activity.

Generally, the population of T-lymphocytes generated by the method of the present invention has the desired purity without further separation, since the cancer cells are lysed by the T-lymphocytes, and the leukocytes that are not activated by the tumor cells with which they are co-cultured do not proliferate. However, if there are residual cancer cells which have not been lysed or other undesired cells in the preparation, these can be removed by known methods to obtain a substantially pure population. If residual cancer cells remain in the sample, these can be removed by any method known in the art, *e.g.*, centrifugation over Ficoll-Hypaque™ gradients. Non-cancerous patient's cells which are not T-lymphocytes can be removed by FACS, using cell surface marker-specific antibodies.

The methods of the present invention encompass CSCTLs which are transformed with a polynucleotide expression vector comprising a coding sequence. The coding sequence may encode any protein having a therapeutic effect particularly a cytokine or growth factor, such as IL-4, IL-2, TNF-α and GM-CSF. Alternatively, or in addition, the coding sequence may encode a protein which enhances the therapeutic effect of the CSCTLs; *e.g.*, a therapeutic protein may enhance the ability of T-lymphocytes to lyse cancer cells, or may prolong the length of time which a CSCTL retains ability to proliferate. This may include an antibody or T-cell receptor binding site, an adhesion molecule, or other cell surface protein to enhance targeting to tumor cells. The sequence encoding a therapeutic protein is operably linked to control sequences for transciption and translation. A control sequence is "operably linked" to a coding sequence if the control sequence regulates transcription or translation. Any method in the art can be used for the transformation, or insertion, of an exogenous polynucleotide into a host cell, for example, lipofection, transduction, infection or electroporation, using either purified DNA, viral vectors, or DNA or RNA viruses. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host cell genome.

The invention further encompasses methods of treating cancer patients, particularly humans. The patient treated is typically the patient from whom the leukocytes and tumor cells were obtained, although treatment of other histocompatible patients is contemplated.

Suitable cancers which can be treated by the methods of the present invention, and from which tumor cells can be obtained for use in generating CSCTLs, include, but are not limited to: brain tumors, such as astrocytoma, oligodendroglioma, ependymoma, medulloblastomas, and Primitive Neural Ectodermal Tumor (PNET); pancreatic tumors, such as pancreatic ductal adenocarcinomas; lung tumors, such as small and large cell adenocarcinomas, squamous cell carcinoma and bronchoalveolarcarcinoma; colon tumors, such as epithelial adenocarcinoma and liver metastases of these tumors; liver tumors, such as hepatoma and cholangiocarcinoma; breast tumors, such as ductal and lobular adenocarcinoma; gynecologic tumors, such as squamous and adenocarcinoma of the uterine cervix, and uterine and ovarian epithelial adenocarcinoma; prostate tumors, such as prostatic adenocarcinoma; bladder tumors, such as transitional, squamous cell carcinoma; tumors of the reticuloendothelial system (RES), such as B and T cell lymphoma (nodular and diffuse), plasmacytoma and acute and chronic leukemia; skin tumors, such as melanoma; and soft tissue tumors, such as soft tissue sarcoma and leiomyosarcoma.

As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and may be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of the treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

The "pathology" associated with a disease condition is any condition that compromises the well-being, normal physiology, or quality of life of the affected individual. This may involve, but is not limited to, destructive invasion of affected tissues into previously unaffected areas, growth at the expense of normal tissue function, irregular or suppressed biological activity, aggravation or suppression of an inflammatory or immunologic response, increased susceptibility to other pathogenic organisms or agents, and undesirable clinical symptoms such as pain, fever, nausea, fatigue, mood alterations, and such other features as may be determined by an attending physician.

A population of CSCTLs may be introduced into a patient in various ways. In one embodiment of the invention, the CSCTLs are implanted at the tumor site. The tumor is resected and the CSCTLs are implanted into the tumor bed. The cells are implanted into the patient during a surgical procedure, using a syringe and needle and are generally allowed to come into direct contact with tumor cells. Thus, as used herein the terms "implant" and "implanted" mean that the cells are placed into or adjacent to the tumor. Preferably, the CSCTLs are in a cell-compatible solution such as isotonic saline/1% albumin or autologous patient plasma, or in a gel made up of synthetic thickeners or clotted autologous plasma. The CSCTLs can be implanted in the proximity

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of a surgically debulked tumor, or a tumor treated by irradiation, chemotherapy, or other appropriate technique. Alternatively, the tumor is not resected, and CSCTLs are implanted at the tumor site. This can be accomplished, *e.g.*, by injection with a syringe and needle directly into the tumor, or by endoscopic delivery to the tumor site.

In another embodiment, the CSCTLs are infused into the patient at a site distant from the tumor. Preferably, infusion is systemic such as by intravenous or peritoneal administration. If infusion is by an intravenous route, preferably an in-dwelling catheter is used. Preferably, the infusion of cells is carried out over a period of time, which may be approximately 10 minutes.

The subjects may have an advanced form of disease, in which case the treatment objective may include mitigation or reversal of disease progression, and amelioration of side effects. The subjects may have had a history of the condition, for which they have already been treated, in which case the objective response will typically include a decrease or delay in the risk of recurrence.

The methods of treatment comprise administering an amount of a pharmaceutical composition containing CSCTLs effective to achieve the desired result, be it palliation of an existing tumor mass or prevention of recurrence. An effective amount is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of the disease, or otherwise reduce the pathological consequences of the disease. An effective amount may be provided in one or a series of administrations.

The effective amount of CSCTLs to be administered will depend upon several factors, such as the route of administration, the condition of the patient, and the desired objective. The term "therapeutically effective" means that the amount of CSCTLs used is of sufficient quantity to ameliorate the cancer. "Ameliorate" denotes a lessening of the detrimental effect of the cancer on the patient. Typically, if administered directly, the amount per administration is related to the size of the tumor. For a tumor of approximately 4 cm in diameter, the amount per administration is generally about 10^8 cells to 10^{10} cells, or more preferably 10^9 to 4×10^9 cells and more typically about 2×10^9 cells. Cells are typically administered once, followed by monitoring of the clinical response, such as diminution of disease symptoms or tumor mass. Administration may be repeated on a monthly basis, for example, or as appropriate.

The various compositions of this invention can be used alone, or in conjunction with other active agents that promote the desired objective, or provide a desirable adjunct therapy. Preferably, the adjunct therapy is not immunosuppressive. For instance, CSCTL therapy should follow irradiation or chemotherapy (if any) after the immunosuppressive activity due to such therapy has diminished. Suitable active agents include the anti-neoplastic drugs, bioresponse modifiers, antibody therapies and effector cells such as those described by Douillard et al. (1986) *Hybridomas* (Supp. 1:5139); or MCL implant therapy described in WO 96/29394; WO 98/(pending; attorney docket 22000-30580.00); WO 98/(pending; attorney docket 22000-30581); and WO 98/(pending, attorney docket 22000-30582.00).

Other adjunct therapies can be found, for example, in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe et al. (1982) *Monoclonal Antibodies in Clinical Medicine*, Academic press, pp. 168–190; Vitatta (1987) *Science* 238:1098–1104; and Winter and Milstein (1991) *Nature* 349:293–299, "Chimeric Toxins," Olsnes and Pihl, *Pharmac. Ther.* 15:355–381 (1981) and "Monoclonal Antibodies for Cancer Detection and Therapy," eds. Baldwin and Byers, pp. 159–179, 224–266, Academic Press (1985). Bioresponse modifiers may also be used, and include, but are not limited to, lymphokines and cytokine such as tumor necrosis factor (TNF), interleukin-2 (IL-2), interleukin-4 (IL-4), granulocyte-macrophage colony stimulating factor (GM-CSF) and γ (gamma) interferons. Other adjunct therapies of interest is Gemsar®.

When the site of delivery is *in situ* to the brain, any adjunct therapeutic agent must be able to cross the blood-brain barrier, for example, by free diffusion or facilitated transport, or by direct physical introduction into the CNS.

Where it is preferable to administer the compositions locally to the area in need of treatment; this may be achieved by, for example, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. A suitable such membrane is Gliadel® provided by Guilford Sciences.

Those in whom the cancer has recurred are especially suitable for treatment with the compositions of this invention. Suitable human subjects for therapy comprise two groups, which may be distinguished by clinical criteria. Patients with "advanced disease" or "high tumor burden" are those who bear a clinically measurable tumor. A clinically measurable tumor is one that can be detected on the basis of tumor mass (e.g., by palpation, MRI, CAT scan, or X-ray; positive biochemical or histopathological markers on their own are insufficient to identify this population). A pharmaceutical composition embodied in this invention is administered to these patients to elicit an anti-tumor response, with the objective of palliating their condition. Ideally, reduction in tumor mass occurs as a result, but any clinical improvement constitutes a benefit. Clinical improvement includes decreased risk or rate of progression or reduction in pathological consequences of the tumor.

A second group of suitable subjects is known in the art as the "adjuvant group". These are individuals who have had a history of cancer, but have been responsive to another mode of therapy. The prior therapy may have included (but is not restricted to) surgical resection, radiotherapy, and traditional chemotherapy. As a result, these individuals have no clinically measurable tumor. However, they are suspected of being at risk for progression of the disease, either near the original tumor site, or by metastases.

This group may be further subdivided into high-risk and low-risk individuals. The subdivision is made on the basis of features observed before or after the initial treatment. These features are known in the clinical arts, and are suitably defined for each different cancer. Features

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typical of high risk subgroups are those in which the tumor has invaded neighboring tissues, or who show involvement of lymph nodes.

The invention further encompasses compositions comprising CSCTLs and a physiologically acceptable buffer and/or matrix. Any buffer known in the art of drug delivery is suitable for use herein. Numerous matrices are known in the art and include, but are not limited to, gels of various kinds including synthetic formuations known in th pharmaceutical arts, clotted plasma, or collagen; hyaluronic acid, and silastic membranes. In the case of peripheral administration, the cells are suspended in an isotonic, physiologically acceptable solution. Typically, these solutions include, but are not limited to, isotonic saline, phosphate buffered saline, or any other neutral buffered saline solution. Typically the compositions are formulated in single dosage formats. Typically, a single dose contains 0.5-10 x 10^9 cells. The cells can optionally be suspended in a suitable, isotonic buffer containing a cryopreservative such as dimethylsulfoxide (DMSO) and stored at temperatures below –70°C for future use. For *in vivo* therapeutic administration, the dosages should be substantially sterile and lack pyrogens. If necessary, the cells can be washed and resuspended in sterile, physiologically acceptable buffer prior to use.

The examples presented below are provided as a further guide to the practitioner, and are not meant to be limiting in any way.

EXAMPLES

Example 1: Induction of a systemic anti-cancer immune response

A biopsy revealed the presence of a glioblastoma multiforme in a pediatric female. Various treatments over the course of 3 years were pursued, including: partial and total resection of the tumor after tumor progression; chemotherapy, consisting of two courses of cyclophosphamide; external beam radiation therapy; high dose chemotherapy consisting of cyclophosphamide and melphalan, followed by autologous bone marrow transplant.

In August of 1995, a cytoimplant of activated third-party leukocytes was placed in the tumor bed during a surgical procedure. This therapy improves patient survival by a process which is thought to involve active immunological response in the treated subject (WO 96/29394). Thirty days after the cytoimplant, surgical resection of the implant was performed due to the appearance of expansion of the lesion by MRI. Pathological assessment of the tumor revealed inflammatory changes, necrosis and a small amount of residual tumor. One month later, the patient received a second cytoimplant.

The cytoimplants were performed essentially as described in WO 96/29394 Three days prior to implantation, a genetically unrelated donor was identified and was leukopheresed to obtain the desired number of leukocytes. Leukopheresis for approximately 2.5 hours routinely provided up to 10 x 10^9 mononuclear cells. At the same time, a unit of blood was obtained from the patient,

and the buffy coat was obtained by centrifugation. The mononuclear cells (PBMC) from the donor and the patient were then obtained by centrifugation over Ficoll-Hypaque™ gradients (density = 1.077). Patient mononuclear cells were then inactivated by treatment with mitomycin-C (MC, Mutamycin) at 10 µg/ml for 1 h at 37°C, and washed to remove excess drug. Donor mononuclear cells were then mixed with the MC-treated patient mononuclear cells at 10:1 to 20:1 ratio in AIM V medium (total cell density = 2×10^6 cells/ml. The cells were dispersed into plastic culture bags (Baxter), and placed at 37°C in a humidified, 5% CO_2/95% air incubator. After a three day incubation, viable cells were recovered by centrifugation, counted, suspended in 4-5 ml of sterile patient plasma, and transported to the operating room. At the time of implantation, calcium gluconate was added to initiate a clot. The clot was then minced in a sterile metal dish and placed into the debulked tumor bed during surgery.

Example 2: Characterization of CSCTLs

In this example, CSCTLs were generated in test cultures from the patient treated as described in Example 1, and the culture conditions were optimized.

First, a stable cell line was established from her tumor. Viable tumor cells from a surgically removed tumor were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS). The cell line was designated PGA-95. Viable tumor cells were also recovered from subsequently removed occipital lobe, and used to generate a second cell line, designated PGA-96. The two cell lines have similar if not identical characteristics.

Studies were conducted thereafter to determine whether the patient had developed detectable tumor-specific immunity. PBMC that had been collected and cryopreserved at the time of the August cytoimplant were cultured in a mixed lymphocyte tumor cell culture (MLTC) with viable, growing PGA-95 cells at varying ratios in RPMI-1640/10% FCS for up to 8 days. No anti-tumor activity was detected. However, when PBMC obtained from the patient in January 1996 were cultured with PGA-95 cells in an identical manner, a strong anti-tumor cell activity was noted. Killing was measured microscopically after staining the remaining cells with crystal violet.

The results are shown in Table 1. Values are expressed as % killing of tumor cells. Virtually 100% of the tumor cells were killed during 7-8 days of co-culture at a PBMC:PGA-95 ratio of 100:1. Similar results were obtained at a 50:1 ratio, and about 50% killing of the tumor cells was observed at 10:1. Importantly, no killing of the PGA-95 cells occurred when unrelated (third-party) PBMC were used in the co-culture instead.

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TABLE 1: Generation of Tumor Reactive Cytotoxic T Lymphocytes (CTL) During Mixed Lymphocyte-Tumor Cell Culture (MLTC)

Lymphocytes	MLTC			
	Patient Tumor		Patient Fibroblast	
	50:1	10:1	50:1	10:1
Patient PBMC (Post-implant)	>90	50	<10	<10
Patient PBMC (Pre-implant)	<10	<10	<15	<10
Unrelated PBMC	<10	<10	<10	<10

In a trial MLTC in which the PBMC were cultured with the autologous cancer cell line PGA-96, high levels of IL-2 (approximately 1-1.5 ng/mL) were produced on days 4-6, falling to much lower levels by day 8. No IL-4 was detected during 8 days of culture. TNF was produced at significant levels only after 6 days, and continued to increase to about 0.5 pg/mL by day 8. The phenotype of the responding cells was determined by flow cytometric analysis, and found to be a mixture of CD3+/CD4+ and CD3+/CD8+ cells. When the PBMC were cultured with a prostatic cancer line (LnCAP) or autologous control cells, IL-2 levels were generally below 0.2 ng/mL, and TNF secretion was not significant.

Figure 1 shows testing for optimal growth conditions. The growth of the tumor-reactive CTL was dependent upon the presence of both IL-2 and tumor antigens in the form of autologous tumor cells. PBMC from the patient were cultured with autologous tumor for approximately 10 days. The cells were collected, washed and replated at 10^5 cells/mL under various conditions. The cells which were cultured in the presence of tumor antigen and IL-2 (diamonds; solid lines) grew to substantially higher cell number than did the cells grown in the absence of IL-2 (diamonds; broken lines), in the presence of IL-2 alone (squares; broken lines), or in the presence of tumor antigen alone (triangles; broken lines). In the latter three culture conditions, cell numbers declined after about 13 days in culture, while cells cultured in the presence of tumor antigen plus IL-2 continued to divide, even after 15 days in culture.

Figure 2 shows testing of growth conditions for CTL raised against other tumor cells. PBMC from the same patient were cultured with U373 cells (Upper panel) or ACBT cells (Lower panel), two unrelated brain cancer cell lines. The cultured cells were then recovered and replated under the conditions shown under the following conditions: tumor antigen and IL-2 (open circles, dashed lines); tumor antigen alone (triangles, dotted lines); IL-2 alone (squares, broken lines); or

neither (diamonds, solid lines). Again, the cells grew most rapidly in the presence of both a source of tumor antigen and IL-2.

The specificity of the generated CTL was determined either by vital dye uptake, or by standard ^{51}Cr release assay (Mishell and Shiigi, eds. *Selected Methods in Cellular Immunology* pp. 128-137 (1980) W.H. Freeman and Co), at an effector:target cell ratio of approximately 50:1. CTL generated against PGA-96 cells efficiently killed both PGA-95 and PGA-96 cells (approximately 30% lysis in about 5 h), or cryopreserved tumor cells that had not been previously cultured. The CTL did not kill autologous PBL or any of a panel of unrelated cancer cell lines, including two glioma cell lines developed from other patients. CTLs prepared using a different tumor cell line specifically lysed those tumor cells but not PGA-96.

Example 3: Expansion of CSCTLs

Preparative amounts of CSCTLs were obtained as follows. Peripheral blood cells were isolated from approximately 5 cc. of whole blood from the patent described in Example 1. Peripheral blood mononuclear cell (PBMC) population was obtained by centrifugation over Ficoll-Hypaque™ gradients. PBMC and tumor cells were co-cultured with autologous, viable tumor cells at 100:1 PBMC:tumor ratio, and a density of PBMC of $2x10^6$ viable cells/ml, in RPMI 1640 medium containing 10% FCS in 12 well flat bottom culture plates. After 10 days, cells were transferred to serum-free AIM-5 medium. Fresh autologous tumor cells and IL-2 (100-200 U/ml) were added. This was repeated about every 10 days or after each culture split.

Figure 3 shows the growth curve of the cells (designated PGA-CTL), and when IL-2 or antigen (Ag) was added to the culture. Cells were expanded over about 30 days to approximately 1 to 2-10 x 10^9 CTL.

Figure 4 shows the specificity characteristics of CTL cells against various targets in a standard 4-hour cytotoxicity assay. CTL cells were raised against three different tumor targets in preparative 30 day cultures. Cytotoxic activity and specificity was compared with LAK cells, obtained by culturing the same patient's PBMC in a medium containing 1000 units of IL-2 for 7 days. The targets were ^{51}Cr-labeled cells at an effector:target ratio of 50:1. Individual target cells were as follows: PGA-96_0, tumor cells taken from the patient and expanded in culture; PGA-96_N, tumor cells taken from the patient and cryopreserved without expanding, thawed just before use in the assay; ACBT and U373, two unrelated brain cancer cell lines; UCI107, BLT-2, LNCAP, K562, four cell lines from other types of cancers. The CTL lines were specific in that they lysed brain cancer cell targets from the same line against which they were raised, but lysed other lines much less effectively. Specific killing of autologous tumor cells by the PGA-CTL was about 30%.

Figure 4 also shows MHC restriction of autologous cell killing. PGA-CTL were tested on labeled PGA-96_0 targets, in the absence or presence of 10 µg monoclonal antibody against a class I or class II monomorphic (shared) determinant. Anti-class I antibody inhibited cytolysis by 31%,

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indicating that some of the CTL in the population are class I restricted. Anti-class II antibody inhibited cytolysis by 43%, indicating that some of the CTL in the population are class II restricted. If these populations are non-overlapping, then 26% is unaccounted for. This may represent tumor specific cytolysis that is not MHC restricted, or incomplete inhibition by the antibody.

Figure 5 shows phenotypic analysis of the cells at two different times of expansion culture. At about day 10, the cells are mostly CD3+ and CD19-, indicating a proponderance of lymphocytes and a paucity of NK cells. At least about 80% are CD4+CD8-, with a small proportion of CD8+CD4- and a small proportion of double negatives. About 15% stain for CD25, indicating presence of high affinity IL-2 receptor. At about day 21, most of the cells are CD8+CD4-, a small proportion is CD4+CD8-, and a smaller proportion is double positive. Thus, the cells undergo a phenotypic shift (probably an outgrowth) of CD4 positive to CD8 positive cells. The remaining CD4+ cells may account for the cytolytic activity that is MHC class II restricted. About 25% stain for CD25, and it is believed that a high proportion of the tumor specific cytolytic cells may reside in this population.

In the more general practice of this invention, the proportion of CD8+ cells in the population is preferably at least about 70%, and more preferably at least about 80%. The proportion of cells that is either CD8+ or CD4+, or both, is preferably at least about 90%. The proportion of CD25+ cells is preferably at least about 15%, more preferably at least about 25% or even 35%. The proportion of tumor-specific cells is preferably at least about 5%, more preferably at least about 10%, and even more preferably at least about 20%. Preferably at least about 20% target cells, more preferably at least about 30% target cells, and still more preferably at least about 40% target cells are killed in a 4 hour culture at an effector:target ratio of 50:1; preferably about 90% and more preferably 98% are killed after 24 hours.

MLTC was used to monitor the systemic anti-tumor cellular immunity in the patient as time progressed. Starting in January 1996, blood samples were collected every 2-3 weeks. In the MLTC assay, 100% of the tumor cells were killed at PBMC:PGA ratios of 100:1 and 50:1, with proportionately less killing at lower ratios. In April, the activity fell to the point where only about 25% of tumor cells were killed at 100:1, and even less at 50:1.

During the period that the CSCTLs were being prepared, the patient's tumor continued its aggressive growth. Several different types of treatment were performed, and the patient's immune system was boosted with a cellular vaccine comprising MLC-activated third-party leukocytes mixed with tumor cells. In late June, MRI detected regrowth near the original tumor site and a second mass on the other side of the midline. The regrowth was resected on about July 1 and the second mass was injected with 2×10^9 of the cultured cells. The second mass was not detected by MRI the following day, and the patient was stable for several weeks. In late August when substantial tumor mass was again apparent, the patient was injected peripherally with 0.05×10^9 ^{111}In-labeled cells. The sequential accumulation of radioactivity near the tumor site was consistent with selective homing of the injected cells. Two separate administrations of about 0.2×10^9 and then

about 0.3 x 10^9 unlabeled cells were made over the weeks following, at a time when the patient's condition was grave. A few days after the last administration, the patient died due to a brain-stem hemorrhage that was concurrent with progressive tumor growth.

In the U.S.A. and other jurisdictions where permitted, all patents, patent applications, articles and publications mentioned herein, both supra and infra, are hereby incorporated into this disclosure by reference in their entirety.

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CLAIMS

What is claimed as the invention is:

1. A method of obtaining a cellular composition enriched for a population of human T-lymphocytes with specific cytotoxic activity against autologous cancer cells, comprising the steps of:

a) obtaining cancer cells from a patient;

b) administering to the patient an immunogenic composition capable of eliciting or boosting a specific immunological response against cancer cells of the patient, wherein the administering is performed before, during or after step a);

c) obtaining a leukocyte population comprising T-lymphocytes from the patient at a time sufficiently after step b) to permit said immunological response to be elicited or boosted in the patient;

d) culturing the leukocytes obtained in step c) with the cancer cells obtained in step a) or their progeny, for a time and under conditions that allow outgrowth of T-lymphocytes with specific cytotoxic activity against cancer cells of the patient; and

e) harvesting the enriched T-lymphocyte population from the cell culture.

2. The method of claim 1, wherein the administering of the immunogenic composition in step b) comprises implanting alloactivated lymphocytes into a tumor bed of the patient, wherein the alloactivated lymphocytes are obtained by collecting lymphocytes from a third party donor and alloactivating them against leukocytes of the patient.

3. The method of claim 1, wherein the administering of the immunogenic composition in step b) comprises implanting alloactivated lymphocytes into a tumor bed of the patient, wherein the alloactivated lymphocytes are obtained by collecting lymphocytes from a third party donor and alloactivating them against leukocytes of another third party donor.

4. The method of claim 1, wherein the administering of the immunogenic composition in step b) comprises administering a cell population to the patient at a site distant from a tumor bed, the cell population comprising cancer cells from the patient or their progeny, and further comprising lymphocytes from a third party donor alloactivated against leukocytes of the patient or another third party donor.

5. The method of any preceding claim, wherein the cancer cells are glioblastoma cells.

6. The method of any preceding claim, wherein the cell population harvested in step e) has one or more of the following properties:

i) wherein the population contains at least about 1×10^9 cells;

ii) wherein the population contains at least about 10^3 fold more cells than the number of leukocytes present at the initiation of the culture in step d);

iii) wherein the population contains at least about 70% CD8+ cells;

iv) wherein the population contains at least about 10% T cells specific for cancer cells from the patient;

v) wherein the population has the property that in a 4 hour cytotoxicity assay conducted with labeled cancer cells from the patient at a ratio of effector:target cells of 50:1, at least about 30% of tumor cells from the patient undergo specific cytolysis; or

vi) wherein the population has the property that in a 24 hour culture with cancer cells from the patient at a ratio of effector:target cells of 50:1, essentially 100% of tumor cells from the patient undergo specific cytolysis.

7. The method of any preceding claim, wherein the cell population harvested in step e) has the property that a measurable proportion homes to the tumor site in the patient upon intravenous sytemic administration.

8. A mixed cell composition enriched for human T-lymphocytes with specific cytotoxic activity against autologous cancer cells, obtainable according to the method of any of claims 1 to 5.

9. The mixed cell composition of claim 8, having one or more of the following properties:

i) wherein the cell composition contains at least about 1×10^9 cells;

ii) wherein the cell composition contains at least about 10^3 fold more cells than the number of leukocytes present at the initiation of the culture in step d);

iii) wherein the cell composition contains at least about 70% CD8+ cells; or

iv) wherein the cell composition contains at least about 10% T cells specific for cancer cells from the patient.

v) wherein the cell composition has the property that in a 4 hour cytotoxicity assay conducted with labeled cancer cells from the patient at a ratio of effector:target cells of 50:1, at least about 30% of tumor cells from the patient undergo specific cytolysis; or

vi) wherein the cell composition has the property that in a 24 hour culture with cancer cells from the patient at a ratio of effector:target cells of 50:1, essentially 100% of tumor cells from the patient undergo specific cytolysis.

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10. The mixed cell composition of claim 8 or claim 9, having the property that a measurable proportion of cells in the composition home to the tumor site in the patient upon intravenous sytemic administration.

11. The mixed cell composition of claim 8, claim 9 or claim 10, which is substantially devoid of interleukin 2 (IL-2).

12. A method of reducing the number of cancer cells in a patient, comprising administering to the patient an effective amount of a composition according to any of claims 8 to 11.

13. The method of claim 12, wherein the cancer cells are glioblastoma cells.

14. The method of claim 12 or claim 13, wherein the composition is administered systemically.

15. The method of claim 12 or claim 13, wherein the composition is administered in the vicinity of a tumor, optionally after surgical resection or partial resection of the tumor.

16. Use of a mixed cell population according to any of claims 8 to 11 in the manufacture of a medicament for treatment of a human body in surgery or therapy.

17. Use of a mixed cell population according to any of claims 8 to 11 in the manufacture of a medicament for reducing the number of cancer cells in a human.

18. Use of a mixed cell population according to any of claims 8 to 11 in the manufacture of a medicament for treating cancer in a human.

19. The use according to claim 17 or claim 18, wherein the cancer is brain cancer.

20. The use according to claim 17 or claim 18, wherein the cancer is glioblastoma.

21. The use according to any of claims 16 to 20, wherein the medicament is formulated for intravenous administration.

22. The use according to any of claims 16 to 20, wherein the medicament is formulated for administration into the bed of a tumor during surgery.

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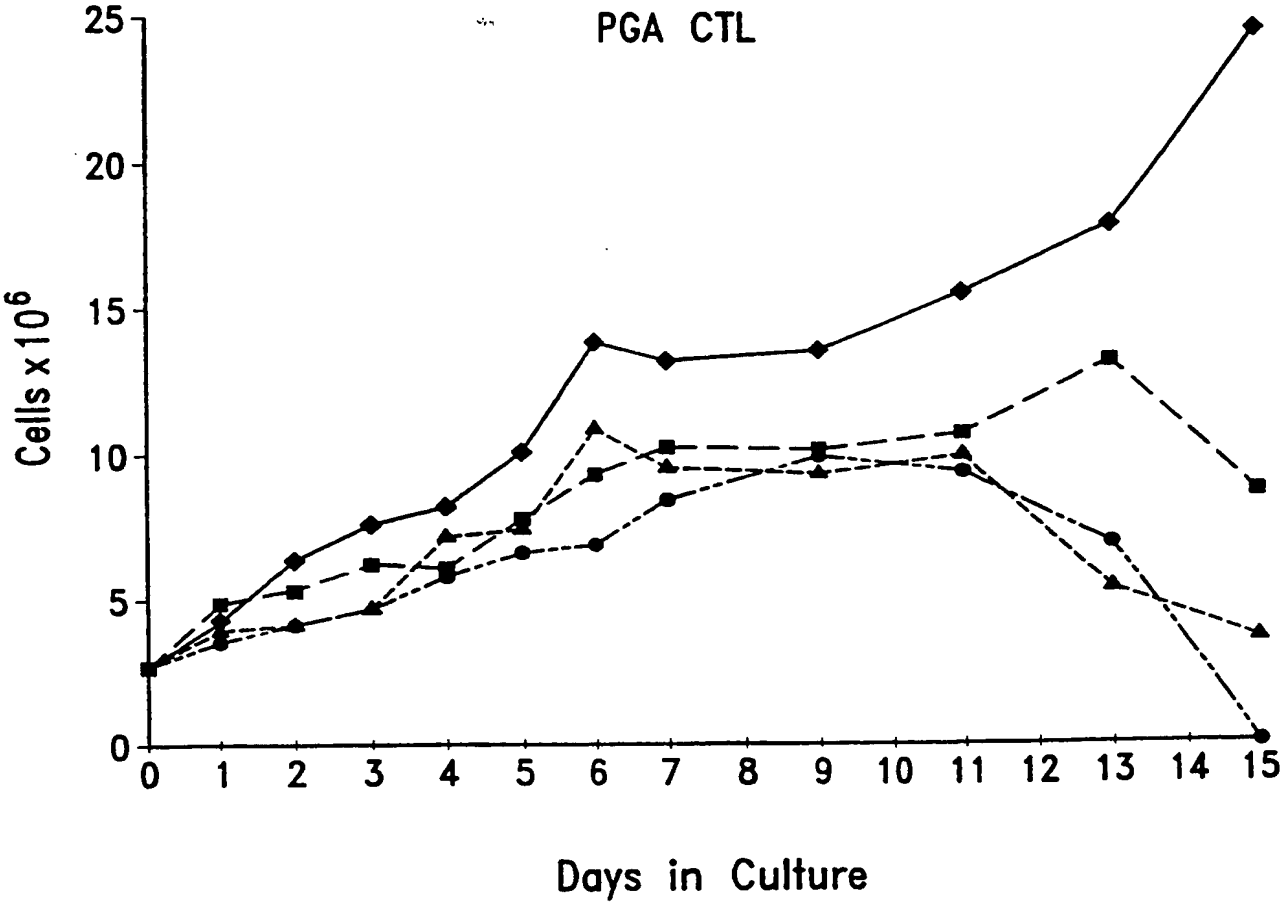

FIG. 1

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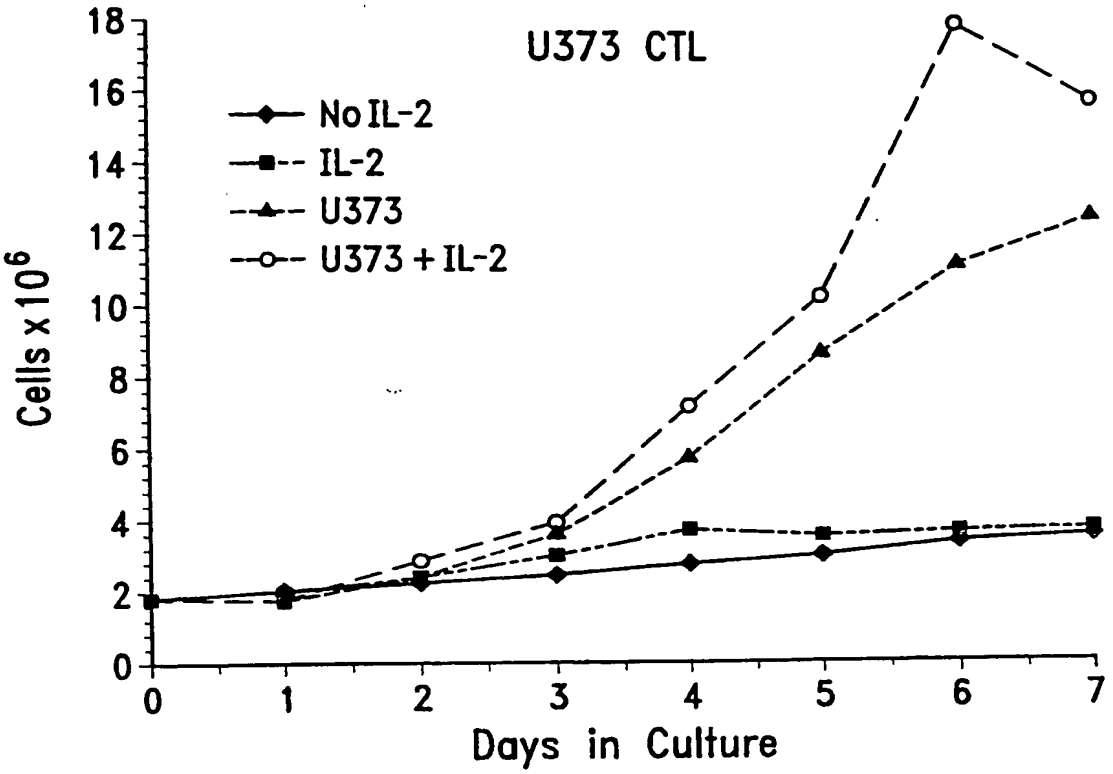

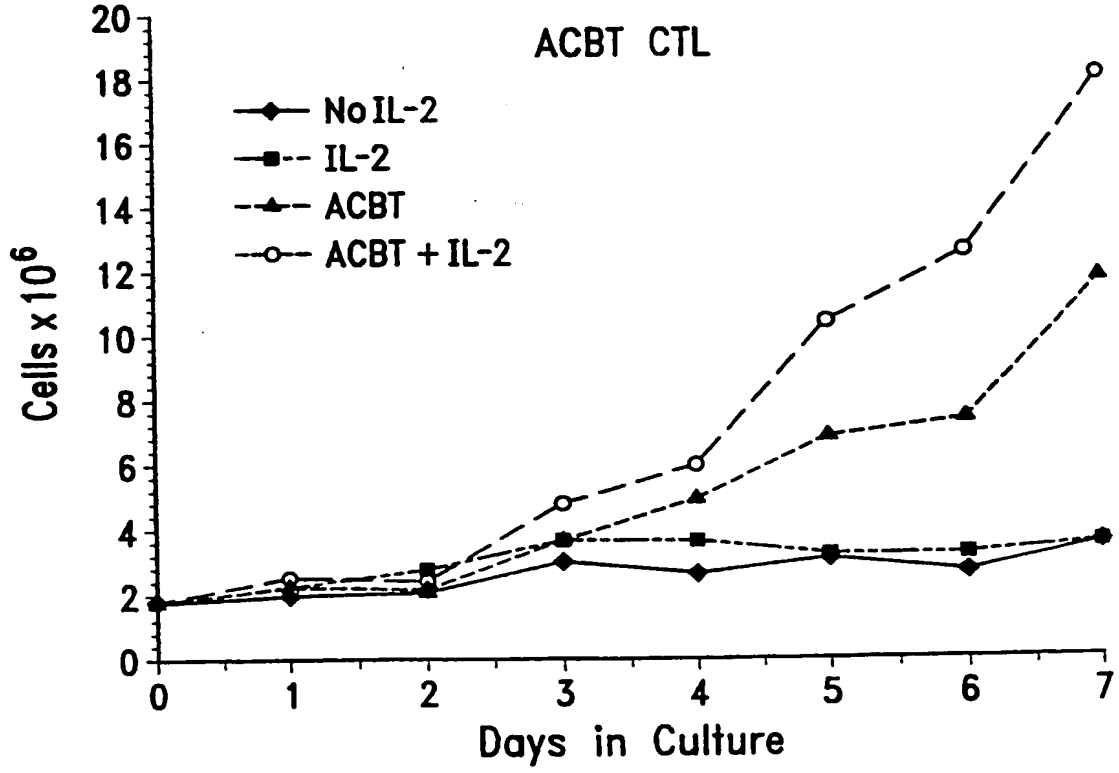

FIG. 2

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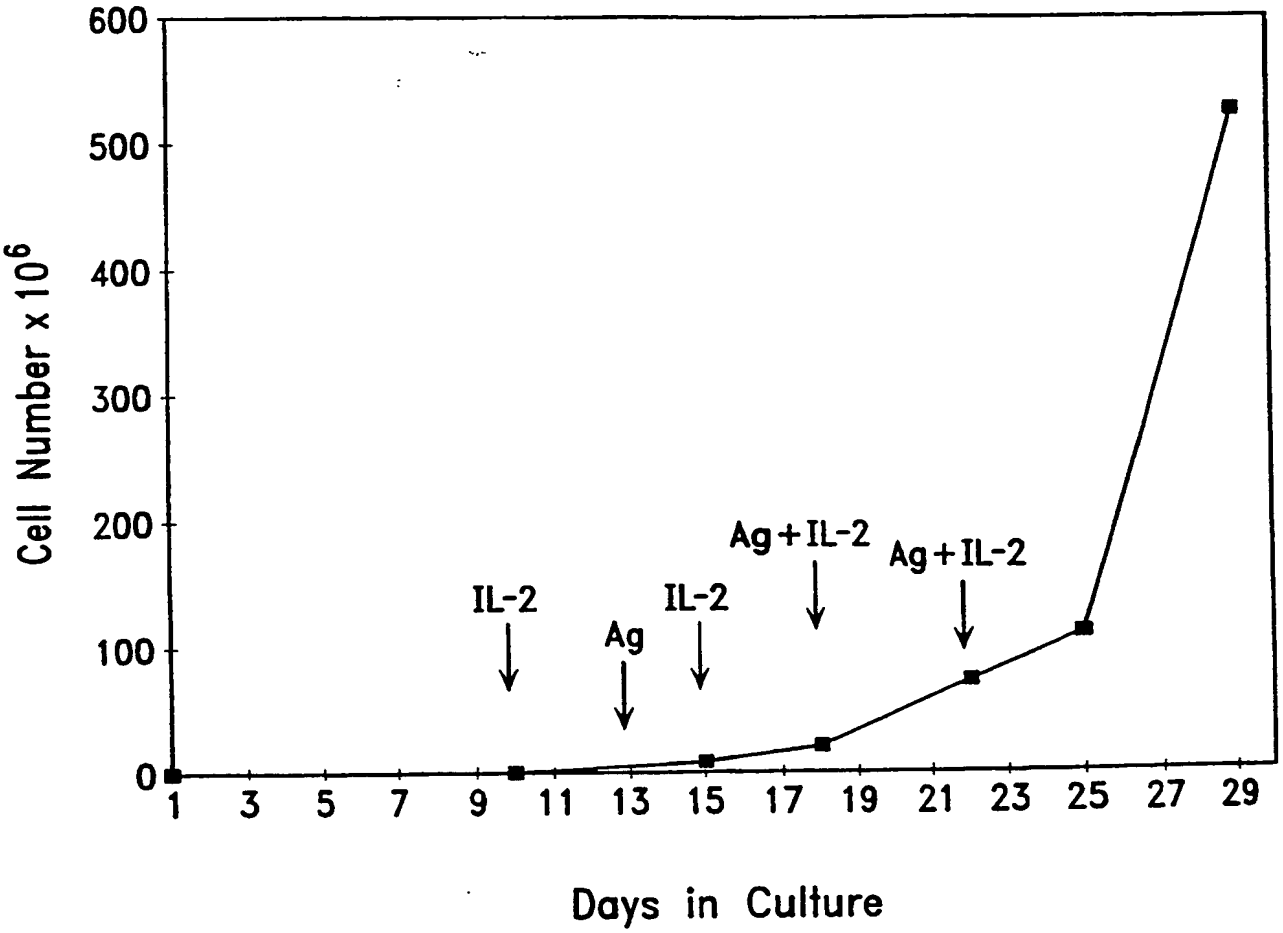

FIG. 3

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	Target @ 50:1							
Effector	PGA-96$_0$	PGA-96$_N$	ACBT	U373	UCI107	BLT-2	LNCAP	K562
PGA96 CTL	[32]	[29]	2	1	8	2	5	17
U373 CTL	8	18	2	[61]	13	[62]	[50]	35
ACBT CTL	0	16	[64]	0	21	11	10	5
LAK	68	63	35	51	28	63	83	58

	None	α Class I	α Class II
PGA-96 CTL [Tested on PGA-96$_0$]	32	22 (31% Inhibition)	18 (43% Inhibition)

FIG. 4

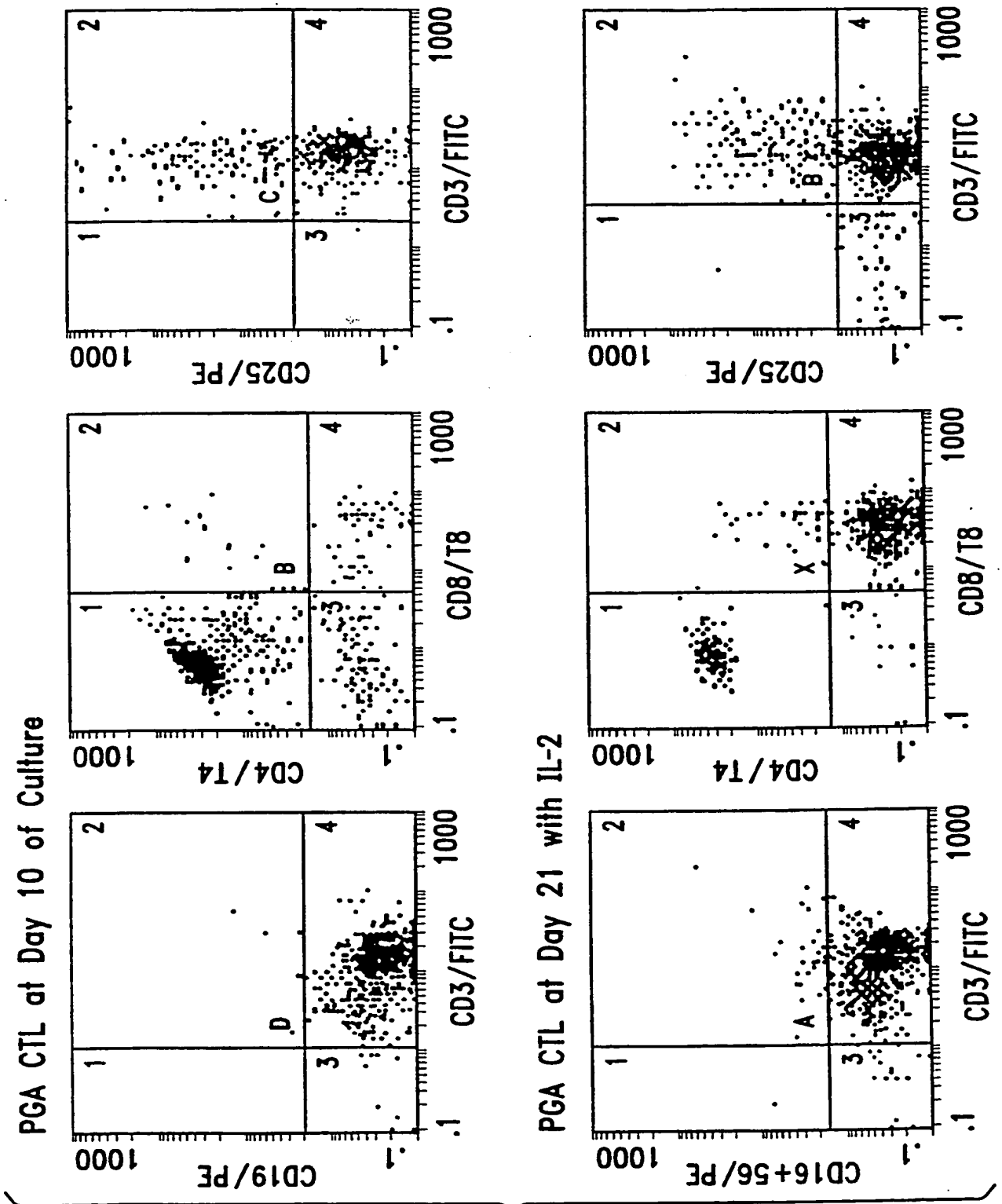

FIG. 5

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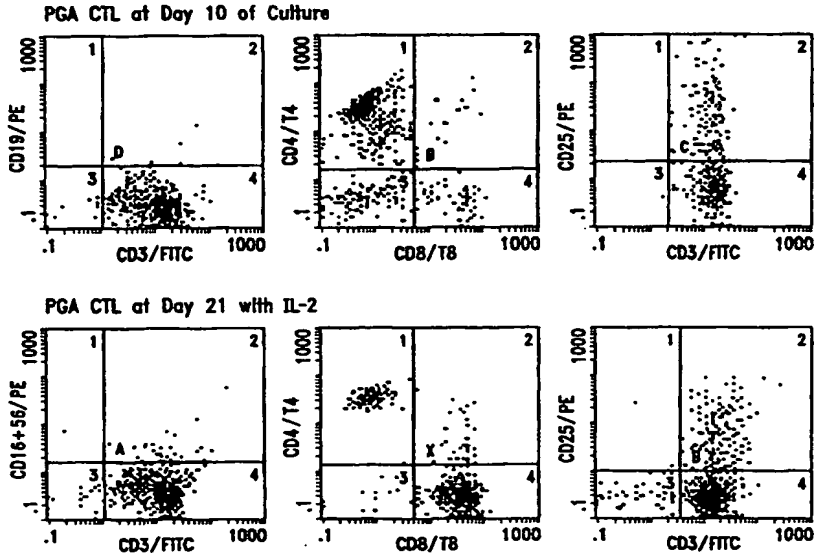
(54) Title: A CELL STRAIN WITH ACTIVATED ANTI-CANCER CYTOTOXIC ACTIVITY



(57) Abstract

The disclosed invention encompasses methods for obtaining compositions comprising cell populations enriched for cancer-specific, cytotoxic T-lymphocytes. The methods comprise the steps of obtaining cancer cells from a patient, obtaining leukocytes from the patient, and co-culturing the cancer cells with the leukocytes to allow outgrowth of a cancer-specific cytotoxic T-lymphocyte (CSCTL) population. In certain embodiments, the method includes the additional step of inducing an anti-cancer immune response in the patient. Substantially pure populations of CSCTLs are provided that are cytotoxic toward autologous cancer cells, and particularly suitable for treating glioblastomas and other solid tumors. This invention further provides methods for treating cancer in a patient using cellular compositions of the invention, and use of cellular compositions for preparing medicaments for cancer treatment.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/08240

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 A61K35/14 A61K45/05

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 690 125 A (INST OF PHYSICAL & CHEMICAL RESEARCH) 3 January 1996 see the whole document ---	1,7,8, 12,14, 16-18,21
X	J.R. SPORN ET AL.,: "Adoptive immunotherapy with peripheral blood lymphocytes cocultured in vitro with autologous tumor cells and interleukine" CANCER IMMUNOLOGY AND IMMUNOTHERAPY, vol. 37, no. 3, 1993, pages 175-180, XP002081415 Sttutgart, DE cited in the application see the whole document --- -/--	1,6,8,9, 12,14, 16-19,21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 October 1998	10/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/08240

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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E	WO 98 16238 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 23 April 1998 cited in the application see the whole document	1-4,7,8, 12,14-18

1

INTERNATIONAL SEARCH REPORT

..ernational application No.
PCT/US 98/ 08240

Box I · Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 7 and 12-15
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 7 and 12-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/08240

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